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Review article

In vitro approaches to assess the hazard of nanomaterials

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ABSTRACT

The rapid development of engineered nanomaterials demands for a fast and reliable assessment of their health hazard potential. A plethora of experimental approaches have been developed and are widely employed in conventional toxicological approaches. However, the specific properties of nanomaterials such as smaller size but larger surface area, and high catalytic reactivity and distinctive optical properties compared to their respective bulk entities, often disable a straightforward use of established *in vitro* approaches. Herein, we provide an overview of the current state-of the art nanomaterial hazard assessment strategies using *in vitro* approaches. This perspective has been developed based on a thorough review of over 200 studies employing such methods to assess the biological response upon exposure to a diverse array of nanomaterials. The majority of the studies under review has been, but not limited to, engaged in the European 7th Framework Programme for Research and Technological Development and published in the last five years. Based on the most widely used methods and/or the most relevant biological endpoints, we have provided some general recommendations on the use of the selected approaches which would the most closely mimic realistic exposure scenarios as well as enabling to yield fast, reliable and reproducible data on the nanomaterial-cell response *in vitro*. In addition, the applicability of the approaches to translate *in vitro* outcomes to leverage those of *in vivo* studies has been proposed. It is finally suggested that an improved comprehension of the approaches with its limitations used for nanomaterials' hazard assessment *in vitro* will improve the interpretation of the existing nanotoxicological data as well as underline the basic principles in understanding interactions of engineered nanomaterials at a cellular level; this all is imperative for their safe-by-design strategies, and should also enable subsequent regulatory approvals.

1. Introduction

Nanotechnology enables the engineering of nanomaterials *i.e.* materials with any external dimension or internal/structural dimension in the nanoscale, with remarkable new physical and chemical properties that differ from their bulk equivalents. This huge potential has led to an increasing growth of research and development activities and created an entire new class of materials which are used in a broad field of applications such as in optics, electronics (*e.g.* for efficient and cost-effective energy storage or their use as semiconductors) (Jariwala et al., 2013), and in the medical field as potential carriers for drug and gene delivery or as diagnostic tools and contrast agents (De Jong and Borm, 2008). However, these new properties and the increasing industrial production have raised concerns about potential adverse effects for human health; thus, a better understanding of cellular consequences upon the direct exposure of (human) cells to these engineered nanomaterials (NMs) is prerequisite for their safe and successful use in any applications.

The number of newly developed NMs with different core materials, sizes, shapes, and coatings is huge (McWilliams, 2016) and expectations from society, consumer and regulatory bodies about their safety are increasing. The characteristics of NMs can be influenced by various physico-chemical parameters, in addition, a proper safety assessment of every nanoform would be extremely cost-intensive and time-consuming. Moreover, the outcomes of animal testing regarding its predictive power for human beings exhibit certain limitations, mainly due to physiological and biochemical species dissimilarities (Shanks et al., 2009). In addition to that, the principle of the 3Rs – Replacement, Reduction and Refinement – has become an increasing public and legal demand which ethically supports the replacement of animal use with

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more human-relevant alternatives that do not rely on *in vivo* testing (Tornqvist et al., 2014). New concepts for efficient, cheaper and evidence-based testing strategies were proposed, based on the use of human primary cells and cell lines (Council NR, 2007). In addition, endpoints for health effects and *in vitro* tests of regulatory interest for conventional chemicals are contained in the Organisation for Economic Co-operation and Development (OECD) and its test guidelines documents (TG) (OECD, 2013a). These *in vitro* tests are rather narrow in their coverage of endpoints: they address genetic toxicity (*e.g.* (OECD, 2015a; OECD, 2014b; OECD, 2014c)), dermal absorption (OECD, 2004) and skin and eye irritation (OECD, 2013b; OECD, 2015b; OECD, 2015c), endocrine disruption (*e.g.* (OECD, 2015d)), and few other selected endpoints. But skin penetration has not been a major concern for NMs while endocrine disruptor effects for NMs are also not currently a focus of research or regulatory concern. Rather, the most relevant *in vitro* protocols for NMs align with the current major routes of NM exposures. Besides dermal (NMs in cosmetic products) and oral (NMs in food products) exposures the effects due to NM inhalation are currently considered to be the most relevant.

Cellular responses have been observed upon exposure to NMs and currently several hypotheses regarding how NMs induce adverse cellular effects exist: (i) *via* oxidative means (the oxidative stress paradigm) which then leads to pro-inflammatory effects (Donaldson et al., 2003), (ii) *via* the fibre paradigm (Dorger et al., 2001; Donaldson and Tran, 2004) (iii) through genotoxicity (Schins and Knaapen, 2007), and (iv) *via* NM dissolution, *i.e.* release of potentially toxic ions and/or other constituents (Bergin and Witzmann, 2013; Braakhuis et al., 2014). The fibre paradigm was highlighted in the paper by Poland et al. (Poland et al., 2008) in which it was shown that multi-walled carbon nanotubes caused granulomas in the peritoneal cavity. This paradigm however, can only be attributed to nanofibres in particular to those with the specific characteristics of high rigidity and high aspect ratio NMs (HARN) (Donaldson et al., 2010).

Other endpoints for NMs which can be examined *in vitro* include those which test for the biological fate of NMs at the cellular or multicellular levels such as size exclusion criteria for given key cell types (Zhu et al., 2013), and adverse effects such as fibrogenicity at these levels of organization (Azad et al., 2013).

The goal of some ongoing research is to unravel modes of action (MOA) of NMs using a plethora of functional assays which are designed to indicate certain MOA relevant to the toxicity and/or fate of NMs and to elucidate biokinetics of NMs, *e.g.* transport through interfaces like air-liquid interface (ALI). It is anticipated that the obtained information on MOA and biokinetics can later be used in weight of evidence analyses or tiered testing schemes in combination with other *in vivo* data, leading to reduction and eventual replacement of *in vivo* tests.

These approaches may help to reduce and/or replace and reduce animal testing according to the 3R strategy. With all these goals, it is critical to use environmentally or occupationally relevant NM concentrations, and to be able to relate these *in vitro* test concentrations to *in vivo* test exposures so that results can be correlated and used in a regulatory context.

A comprehensive review about the relevance of *in vitro* nanotoxicological studies in hazard assessment of NMs has been provided by Park and colleagues in 2009 (Park et al., 2009) where comparison of different cell types and exposure duration was discussed, along with, among others, dose response analysis and potential artefacts in the most commonly used nanotoxicological assays. Since then many new studies have been reported and this review has been developed based on an in-depth summary of over 200 literature reports on the assessment of biological hazard of NMs *in vitro*. The basis for the selection of the selected and relevant results, protocols, and guidance documents, where chosen from the project “A common European approach to the regulatory testing of Manufactured Nanomaterials” (NANoREG) (data deliverables from WP2, WP3, WP4 and WP5; www.nanoreg.eu; (Joint Research Centre, JRC, 2016)), together with the OECD working party of manufactured NMs (WPMN) activities. Peer reviewed publications from other the 7th framework programme for research and technological development (FP 7) projects and US research programmes have been included. However, as some aspects were not comprehensively covered in the above-mentioned literature pool, the authors have searched for other relevant studies employing the publicly available search tools (Web of Science, Pubmed, Google scholar). The aim of this overview is to provide general recommendations for researchers employing *in vitro* assays for assessment of NM hazard based on an extensive literature study. All the recommendations proposed in this manuscript have been developed based on the authors' perspective on the existing literature data on this matter, as well on their experiences with *in vitro* studies for regulatory submissions. Therefore, this overview of the most widely used methodological approaches can serve as a basis for future research directions including thoughts about reported pitfalls for some of the methods and/or approaches.

2. General considerations for *in vitro* test methods

2.1. Nanomaterials

The description of the source of NMs and characterization data has to be given with sufficient detail, including a thorough characterization of both the pristine materials as well as *in situ* (before, during and after the experiments). Additionally, the details of any dispersions methods used for the experiments need to be reported (*discussed in more details in the subchapter 2.3 Dose metrics*).The majority of the reviewed *in vitro* studies report the primary sizes of NMs (transmission electron microscopy (TEM), the hydrodynamic diameter (dynamic light scattering (DLS)) and the zeta potential in water, and/or phosphate-buffered saline (PBS) and/or cell culture medium (Stoehr et al., 2011; Anguissola et al., 2014; Huo et al., 2015a; Shannahan et al., 2015), and specific surface area (Huk et al., 2014; Armand et al., 2016a). In addition to DLS, in some cases, nanoparticle tracking analysis (Di Cristo et al., 2016), differential centrifugal sedimentation (Monopoli et al., 2011; Wan et al., 2015) and photon cross correlation spectroscopy (Gliga et al., 2014) are being used. Regarding light scattering techniques, careful data interpretation is required as agglomeration and sedimentation can occur simultaneously, particularly in the cell culture medium. Particle agglomeration in the cell medium can be, depending on the NM type, investigated by *e.g.* ultraviolet–visible spectroscopy (UV–Vis) (Gliga et al., 2014). Specific surface area can be determined by nitrogen adsorption (Huk et al., 2014); however this approach requires relatively high material masses and is carried out on a powder sample, which is not always feasible in nanotoxicological studies. Depending on the type, inductively coupled plasma (ICP) techniques including ICP-optical emission spectroscopy (-OES) and ICP-mass spectrometry (MS) can be applied for mass concentration of the metal, and UV–Vis for size determination of plasmonic NMs such as silver or gold nanoparticles (NPs) (Stoehr et al., 2011; Gliga et al., 2014; Pang et al., 2016). For soluble NMs, *e.g.* silver NPs or zinc oxide, kinetics of dissolution in cell culture media needs to be assessed over time (Huk et al., 2014; Mu et al., 2014). Noteworthy, transformations of NMs in time (particularly when stored in suspensions) has been suggested as one of the most significant contributors to the contradictory *in vitro* toxicity results observed in the literature for identical NMs; hence, aging needs to be addressed in parallel with the assessment of effects (Izak-Nau et al., 2015). Surface reactivity of NMs in a cell free environment can be measured by the electron spin resonance (ESR) technique; briefly, presence of free radicals can be detected by using so called spin traps, reagents that form adducts to stabilize the radicals, which then exhibit a paramagnetic resonance detectable by spectroscopy (Monopoli et al., 2011). Also, there are other techniques for detection of ROS/free radicals, for instance *via* cytochrome *c* reduction (Dikalov and Harrison, 2014), ferric-reducing ability of serum (FRAS) assay and dichlorofluorescein assay (Pal et al., 2014).